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14. ABSTRACT This proposal is concerned with the functional role of merlin in arachnoidal and meningioma cells. In year 1, we had developed meningioma-specific NF2 model systems. Now, we have finished characterizing the phenotypic effects caused by merlin loss in meningioma cell lines. We have shown that merlin loss enhances loss of contact inhibition of growth and increases anchorage independent growth. Loss of merlin also increases entry into the S-phase of the cell cycle and this effect is associated with an increase in transcript and protein levels of cyclin E1. We also find that merlin loss also decreases the apoptotic rates in meningioma cell lines. Finally, we have initiated an analysis to identify downstream targets of merlin by microarrays. In the last year of funding, we anticipate identifying and characterizing downstream effectors of merlin in meningiomas.						
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INTRODUCTION

Neurofibromatosis 2 (NF2) is a cancer predisposition syndrome that is phenotypically characterized by the presence of multiple benign brain tumors, primarily schwannomas and meningiomas. Intracranial meningiomas, often multicentric, occur in ~50% of NF2 patients and are associated with an increased risk of mortality. The majority of studies evaluating the mechanism of action of the NF2 gene product, merlin, have used cell lines unrelated to NF2 target cells. Since tumor suppressor function is cell-type specific, the relevance of these studies to the function of merlin in meningiomas is unclear. Additionally, conditional NF2 mouse modeling studies and genotype-phenotype correlation studies suggest mechanistic differences in the function of NF2 between schwannomas and meningiomas. The purpose of this study is to develop meningioma-specific NF2 model systems and to use these model systems to investigate the specific tumor suppressive function of NF2 in meningioma development. Our strategy is to isolate and immortalize human meningioma and arachnoidal cell lines, characterize the expression of merlin in these *in vitro* systems and to engineer merlin-deficient or merlin-expressing meningioma and arachnoidal cells in the relevant genetic backgrounds. These NF2 model systems will be used to dissect the pathways and mechanisms by which merlin expresses its growth suppressive effects specifically in meningioma tumorigenesis.

BODY

Outlined below is a summary of the research accomplishments associated with each task outlined for year 2 in the approved Statement of Work. Tables and Figures related to the text are included in the Supporting Data Section. Appendix 1 includes a recent publication from my laboratory and we specifically mention Appendix 1 when referring to a figure in this manuscript. For example, (Figure 1 in Appendix1) would imply Figure 1 in the attached manuscript in Appendix 1 while (Figure 1) refers to the Supporting Data Section.

Task 1. Characterize merlin expression in immortalized meningioma cell lines (Months 1- 24).

- a. Three additional immortalized meningioma cell lines will be generated using telomerase alone or in conjunction with the human papillomavirus E6/E7 oncogene (Months 1-6).

This task was described in detail in the Year 1 Annual Report. Briefly, we generated a total of nine meningioma and three arachnoidal cell lines in my laboratory.

- b. Merlin-deficient cell lines will be chosen using western blot analysis (Months 4-7).

This task was described in detail in the Year 1 Annual Report. Briefly, KT21MG1 was identified as a merlin deficient meningioma cell line, and SF6717 was identified as a merlin positive meningioma cell line. SF6717 is a new cell line established in our laboratory that has been renamed MENII-1 to reflect the fact that it is a meningioma cell line derived from a WHO Grade II tumor. In the rest of this report, we refer to this cell line as MENII-1. We have also described a novel splice site mutation in the KT21MG1 meningioma cell line.

- c. Vector-control and merlin-expressing stable clones will be generated in appropriate meningioma cell lines (Months 6-12).
 - d. The expression of merlin will be assessed in individual stable clones and clones expressing high levels of merlin will be chosen (Months 11-14).

These tasks were described in detail in the Year 1 Annual Report. Briefly, we expressed wild-type NF2 and three specific mutants of merlin (L64P, S518A and S518D) in the KT21MG1 cell line using the Tet-On Advanced Inducible Gene Expression System. Even in the absence of doxycycline, we had some expression of merlin suggesting leakage of the Tet-On System. Therefore, we subcloned wild type NF2 and the three mutants of merlin (L64P, S518A and S518D) into the retroviral vector, pBABE-Hygro. We have generated stable cell populations in KT21MG1 that constitutively express either vector only (KT21MG1-control), wild type NF2 (KT21MG1-NF2) or the L64P, S518A and S518D mutants of NF2. We show the expression of merlin in KT21MG1-NF2 cells by western blot analysis and immunofluorescence (Figure 1B,D).

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- e. *Functional in vitro assays for cell proliferation, apoptosis, motility/invasion and survival will be performed (Months 15-24).*

The experiments performed for this task are described with Task 2d below.

Task 2. Establish an in vitro model of merlin loss in human arachnoidal cells and evaluate the consequent changes in cell growth, motility and survival (Months 1-36).

- a. *Chemically synthesized siRNA for merlin will be obtained and the efficacy of these siRNA to downregulate merlin will be tested using transient transfections (Months 1-6).*
- b. *Merlin-specific siRNA plasmid will be constructed and stable clones expressing merlin siRNA will be generated in immortalized arachnoidal cells (Months 7-11).*
- c. *The extent of downregulation of merlin will be evaluated in individual stable clones and the clone with the greatest level of knockdown of merlin will be chosen (Months 12-14).*

These tasks were described in detail in the Year 1 Annual Report. Briefly, we chose NF2siRNA2 and NF2siRNA3 to achieve stable suppression of NF2 in arachnoidal (AC1) and MENII-1 meningioma cell lines. As shown in the last report, among the two effective siRNAs, NF2siRNA3 was slightly more effective than NF2siRNA2 and therefore we chose to use NF2siRNA3 for generating stable cell populations. Stable cell populations expressing either NF2 siRNA (MENII-1-NF2 siRNA or AC1-NF2 siRNA) or a non-specific target siRNA (MENII-1-Control or AC1-Control) were generated. NF2 transcript levels were 5.6-fold lower in MENII-1-NF2 siRNA1 cells when compared to MENII-1-Control cells using quantitative PCR (Figure 1A). Protein levels of merlin were undetectable in MENII-1-NF2 siRNA and AC1-NF2 siRNA cells by western blot analysis and immunofluorescence revealed loss of merlin staining in AC1-NF2 siRNA and MENII-1-NF2 siRNA cells (Figure 1B,C). Thus, NF2 siRNA was effective at stably suppressing the expression of endogenous merlin.

- d. *Functional in vitro assays for cell proliferation, apoptosis, motility/invasion and survival will be performed on the merlin deficient arachnoidal cells (Months 15-24).*

We next assessed the effect of merlin loss on the growth properties of our three paired meningioma and arachnoidal cell lines described above (AC1-control and AC1-NF2 siRNA; MENII-1-control and MENII-1-NF2 siRNA; KT21MG1-control and KT21MG1-NF2). All three cell lines have more pronounced loss of contact dependent inhibition of growth in the absence of merlin (AC1-NF2 siRNA, MENII-1-NF2 siRNA, and KT21MG1-control) when compared to the presence of merlin (AC1-control, MENII-1-control, and KT21MG1-NF2 siRNA) (Figure 2). This is consistent with previous results suggesting that merlin has a direct effect on loss of contact dependent inhibition of growth (1).

Merlin loss also promoted colony formation in soft agar. The number of colonies in the control cell lines varied depending on the type of cell line. For example, KT21MG1 is derived from a malignant meningioma and is lacking endogenous NF2 and had the largest number of colonies. A greater number of larger colonies were observed in the absence of merlin (AC1-NF2 siRNA, MENII-1-NF2 siRNA, and KT21MG1-control) when compared to the presence of merlin (AC1-control, MENII-1-control, and KT21MG1-NF2 siRNA) in all cell lines tested (Figure 3). Thus, merlin was having a direct effect on the tumorigenicity of meningiomas, consistent with a direct role in meningioma tumor formation. This data is also important for performing Task 2e in Year 3.

In order to determine effects of merlin on cell cycle progression we measured incorporated BrdU and 7-AAD by means of flow cytometry. Interestingly, loss of merlin resulted in a significant increase in the percentage of cells that incorporate BrdU (Figure 4). This effect was the most pronounced in the arachnoidal cells where the percentage went from 16% to 55%. This increase in the number of cells in the S-phase was associated with a concomitant decrease in the G0-G1 and G2 phases of the cell cycle. These results indicate that merlin function as a tumor suppressor by targeting the G0-G1 to S phase cell cycle check point in meningiomas (Figure 5).

We also analyzed the effect of merlin loss on apoptosis in the presence and absence of the apoptotic reagent, Staurosporine (Stsp). Stsp is a pan-tyrosine kinase inhibitor that induces apoptosis in meningioma cell

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lines. The percentage of cells undergoing apoptosis increased from 18.1% to 43.6% in MENII-1-control cells (Figure 6). In the absence of merlin, this rate was considerably decreased both in the absence (18.1% to 6.9%) and presence (43.6% to 27.6%) of 20 nM Stsp. Thus, merlin loss has an effect on the apoptotic rate in meningioma cells by reducing the rate of spontaneous apoptosis and also by enhancing the resistance of meningioma cells to Stsp-mediated resistance. While the effects of merlin on apoptosis have previously been reported for schwannomas (2), there has been no study implicating an effect of merlin on meningiomas.

Cyclin E1 regulates G0-G1 to S transition (3, 4) and is a potential downstream target of Merlin. Therefore, we performed quantitative PCR using primers specific for cyclin E1 to assess transcript levels in MENII-1 cells. In the absence of merlin, cyclin E1 transcript levels were elevated by 2.5-fold (Figure 7A). We performed western blots using a monoclonal antibody specific for cyclin E1 to assess protein levels. Cyclin E1 protein levels were elevated in the absence of merlin (AC1-NF2 siRNA, MENII-1-NF2 siRNA, and KT21MG1-control) when compared to the presence of merlin (AC1-control, MENII-1-control, and KT21MG1-NF2 siRNA) in all cell lines tested (Figure 7B).

- e. *The in vivo ability of these cell lines to form tumors will be tested and the resultant tumors will be characterized (Months 27-36).*

To be completed in Year 3 of funding

Task 3. Identify and characterize downstream target genes that are regulated by merlin in meningioma and arachnoidal cells (Months 18-36).

- a. *Two SAGE libraries will be constructed and sequenced from the vector-control and merlin-deficient arachnoidal cells (Months 18-21).*
- b. *Two SAGE libraries will be constructed and sequenced from the vector-control and merlin-expressing meningioma cells (Months 21-25).*

We had initially planned to use Serial Analysis of Gene Expression to identify differentially expressed genes. SAGE library development involves high sequencing costs that we were planning to perform in collaboration with the Cancer Genome Anatomy Project (CGAP). However, because of changes in CGAP, this is not possible any more. In the past two years, our laboratory has become experienced at performing microarray analysis. We have recently published results from a microarray analysis of primary meningioma tumors (5). Microarray is a technology comparable to SAGE, and analyzes the transcriptomes of cell lines of tumors. It is more cost-effective and has a higher throughput. We have initiated a microarray analysis of our paired cell lines by isolating mRNA from these cell lines. We have performed quality tests on the mRNA using the RNA 6000 Nano kit (Agilent technologies) and are currently generating biotin-labeled cRNA probes for hybridization to the U133 Plus 3.0 microarrays (Affymetrix). This hybridization will be performed at the Gladstone Center Core Facility in the next month.

- c. *Bioinformatics will be performed to analyze the SAGE data and to functionally classify differentially expressed genes (Months 26-29).*
- d. *Quantitative PCR analysis will be initiated on select target genes affected by merlin (Months 30-36).*

To be completed in Year 3 of funding.

In summary, we have successfully completed all the proposed tasks for year 2 of this three-year proposal. We have now generated meningioma specific NF2 model systems and have characterized their functional properties in great detail. In the next year, these systems will be used for *in vivo* functional studies and to identify downstream effectors of NF2.

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KEY RESEARCH ACCOMPLISHMENTS

- 1) We have successfully generated and characterized paired human meningioma cell lines where the only difference is expression of merlin using either RNA interference or tetracycline-inducible system or retroviral mediated gene transfer.
- 2) We have used these paired cell lines to show that absence of merlin causes loss of contact inhibition of growth and enhanced anchorage independent growth in soft agar.
- 3) We have analyzed the cell cycle parameters using these paired cell lines and have shown that merlin loss causes an increase in S-phase entry and a concomitant decrease in G0-G1 and G2 populations.
- 4) We have shown that merlin loss is associated with an increase in transcript and protein levels of cyclin E1.
- 5) Merlin loss has an effect on the apoptotic rates in meningioma cell lines. Merlin loss causes a decrease in spontaneous apoptotic rates and also increases resistance to Stsp-mediated apoptosis.

REPORTABLE OUTCOMES

- 1) We have also generated three sets of paired meningioma cell lines where the only difference is the expression of merlin. These are KT21MG1-Vector and KT21MG1-Merlin; SA2-ControlsiRNA1 and SA2-NF2siRNA3; and SF6717-ControlsiRNA1 and SF6717-NF2siRNA3.
- 2) We have characterized, in great detail, the phenotypic effects of merlin loss on these cell lines
- 3) We used the results of this research, specifically the *in vitro* model systems, to obtain a concept award from the DOD neurofibromatosis program. The proposed research will complement the current study.
- 4) We have submitted a research publication for peer review using the data described in this report.

CONCLUSION

The aim of this proposal is to develop meningioma-specific NF2 model systems and to utilize these systems to investigate the tumor suppressive functions of NF2 in meningiomas. Year 1 of this proposal was focused on characterizing and generating the relevant model systems. The current phase was focused on characterizing the functional properties of these model systems. In the case of three paired human meningioma cell lines, we have assessed the detailed phenotypes resulting from loss of merlin. In all three cases, merlin resulted in loss of contact dependent inhibition of proliferation, enhanced anchorage dependent growth and greater S-phase entry. Elevated cyclin E1 levels are at least partially responsible for the increased S-phase entry. In addition, we have discovered that merlin has a direct impact on apoptosis in meningioma cell lines. This is a novel finding and will be investigated further in Year 3. We have initiated microarray analyses on these cell lines and will use this to discover downstream transcriptional effectors of meningiomas. These model systems can also be used in the future to test potential novel therapeutics against NF2.

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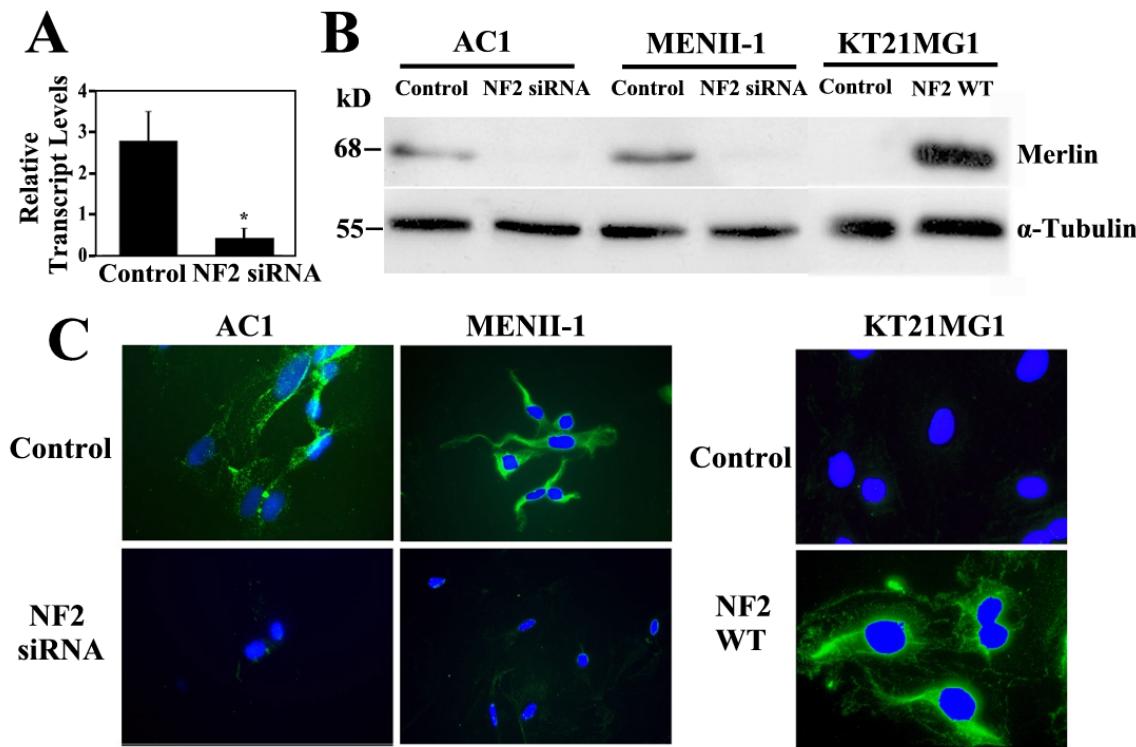


Figure 1. *In vitro* model systems of merlin expression in human arachnoidal and meningioma cell lines. Endogenous merlin was silenced in MENII-1 cells were infected with retroviruses generated using pSUPER.retro.neo-NF2 siRNA or pSUPER.retro.neo-control, and stable cell populations were selected using G418. A, NF2 transcript levels were measured by quantitative PCR and showed a significant reduction in MENII-1-NF2 siRNA cells compared to MENII-1-Control cells. Error bars correspond to mean \pm SE of three independent experiments. B, Western blot analysis of MENII-1 cell lysates to detect merlin was performed. Levels of α -tubulin were determined in the same samples as loading control. Immunoblot of one representative experiment of three with similar results is shown. C, Immunofluorescence using the A19 polyclonal antibody against merlin (Santa Cruz Biotechnology, A-19) shows the presence of cytoplasmic staining in controls and its absence in NF2 siRNA treated cells. D, Immunofluorescence with the A19 antibody showing absence and presence of merlin staining in KT21MG1-Control and KT21MG1-NF2 cells respectively. Merlin immunolabeling is shown in green, DAPI counterstaining of the nuclei is shown in blue.

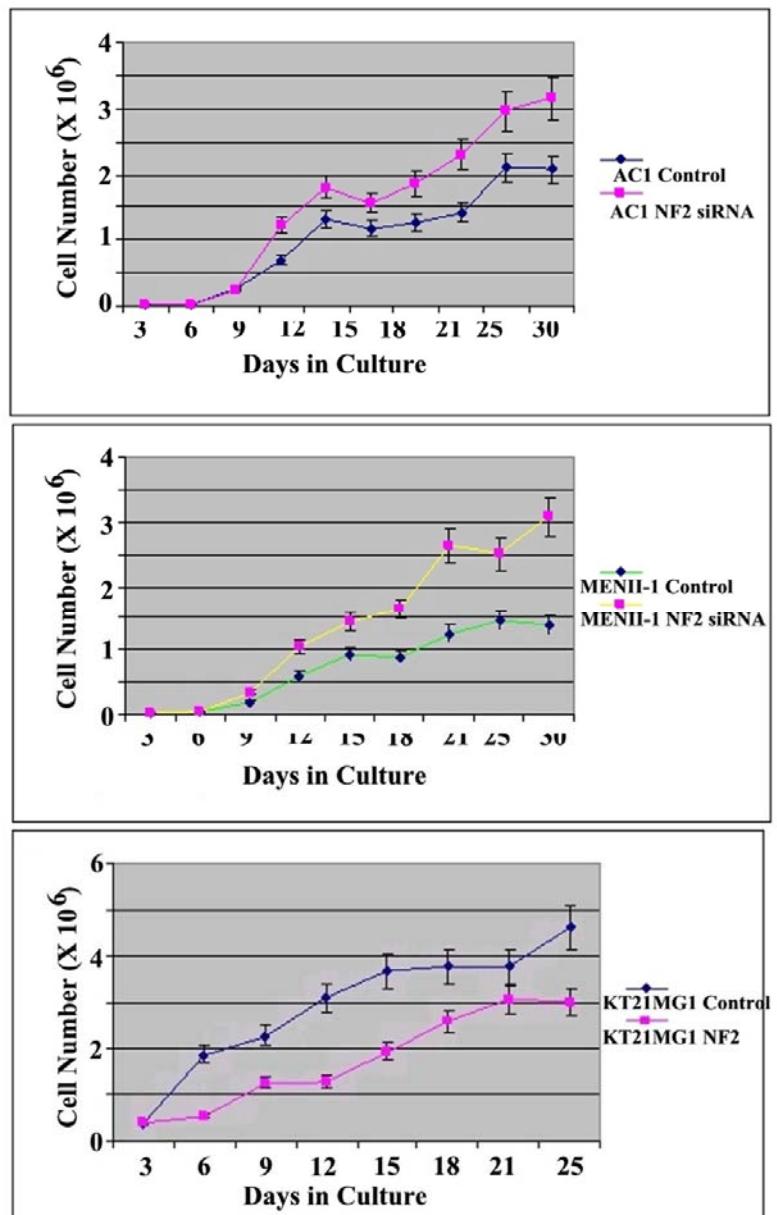


Figure 2. Loss of merlin results in more pronounced loss of contact dependent inhibition of growth. Growth curves of cells with and without merlin expression was measured by plating 20,000 cells in 24 well plates and counting cells from 3 wells at 3, 6, 9, 12, 15, 18, 21, 25 and 30 days. In all cases, the absence of merlin either due to suppression by siRNA (AC1 and MENII-1 or due to lack of endogenous merlin (KT21MG1) was associated with enhanced growth especially under confluent conditions.

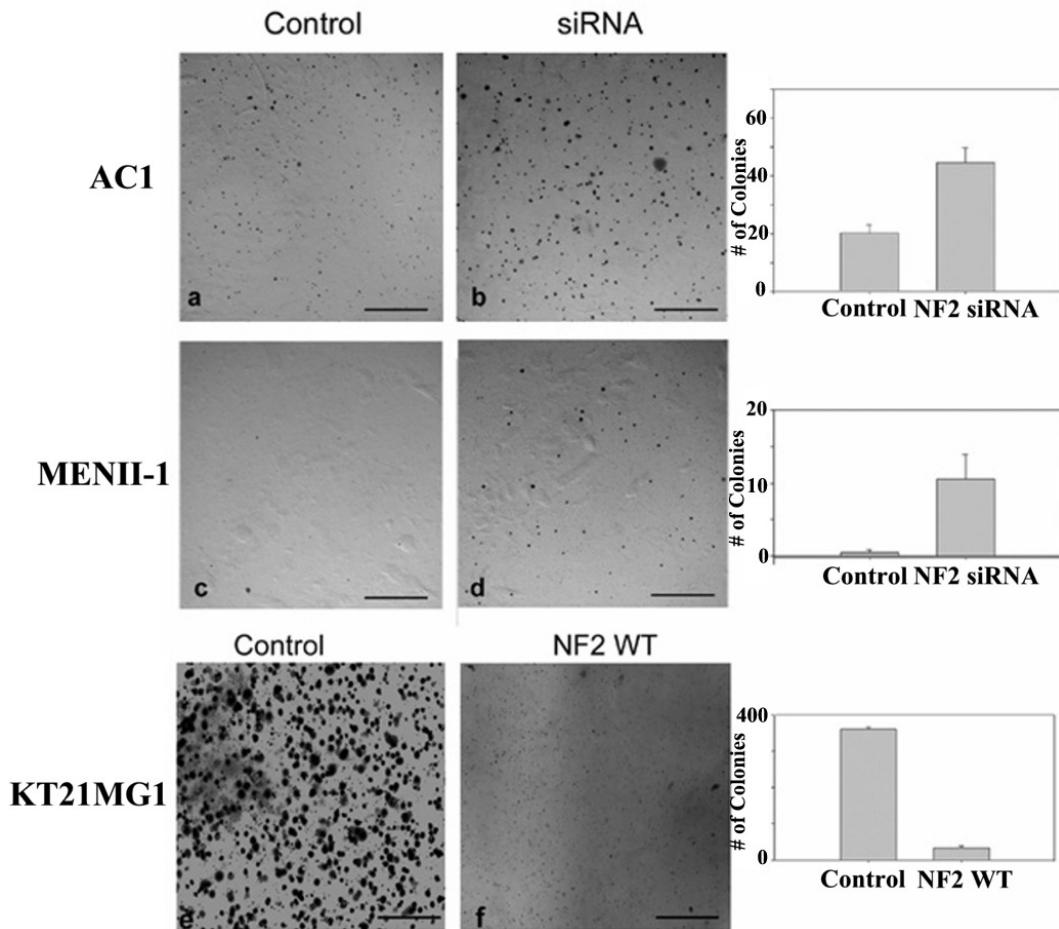


Figure 3: Merlin loss promotes colony formation in soft agar. To assess colony growth in soft agar, 50,000 cells were plated in DMEM in 0.4% low melting temperature agarose upon a layer of 0.8% agarose. After 8 weeks, colonies were stained with 0.005% crystal violet and colonies larger than 100 μm diameter were scored by counting under a microscope. Merlin loss was associated with a greater number of larger cases in all three paired cell lines.

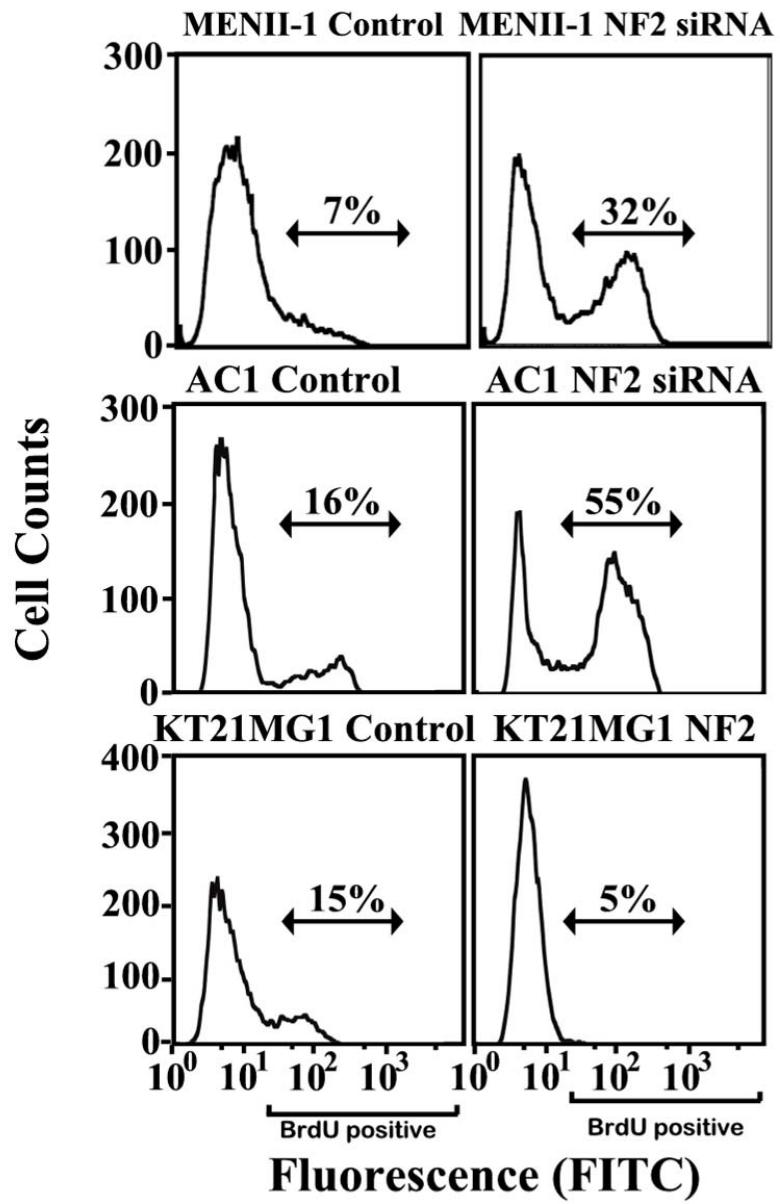


Figure 4. Merlin loss enhances cell proliferation by increasing S-phase entry. Cells (70-80% confluent) were incubated with 1mM bromodeoxyuridine (BrdU) for 3 h and processed for staining with FITC conjugated anti-BrdU and 7-AAD (for total DNA) using the FITC-BrdU Flow Kit (BD Biosciences) following manufacturer's instructions. Flow cytometry was performed on a Becton Dickinson FACSCalibur machine. For each experiment, 10,000 events were counted. Merlin loss in all three paired cell lines was associated with a dramatic increase in BrdU incorporation suggesting that merlin was affecting S-phase entry in meningioma cell lines.

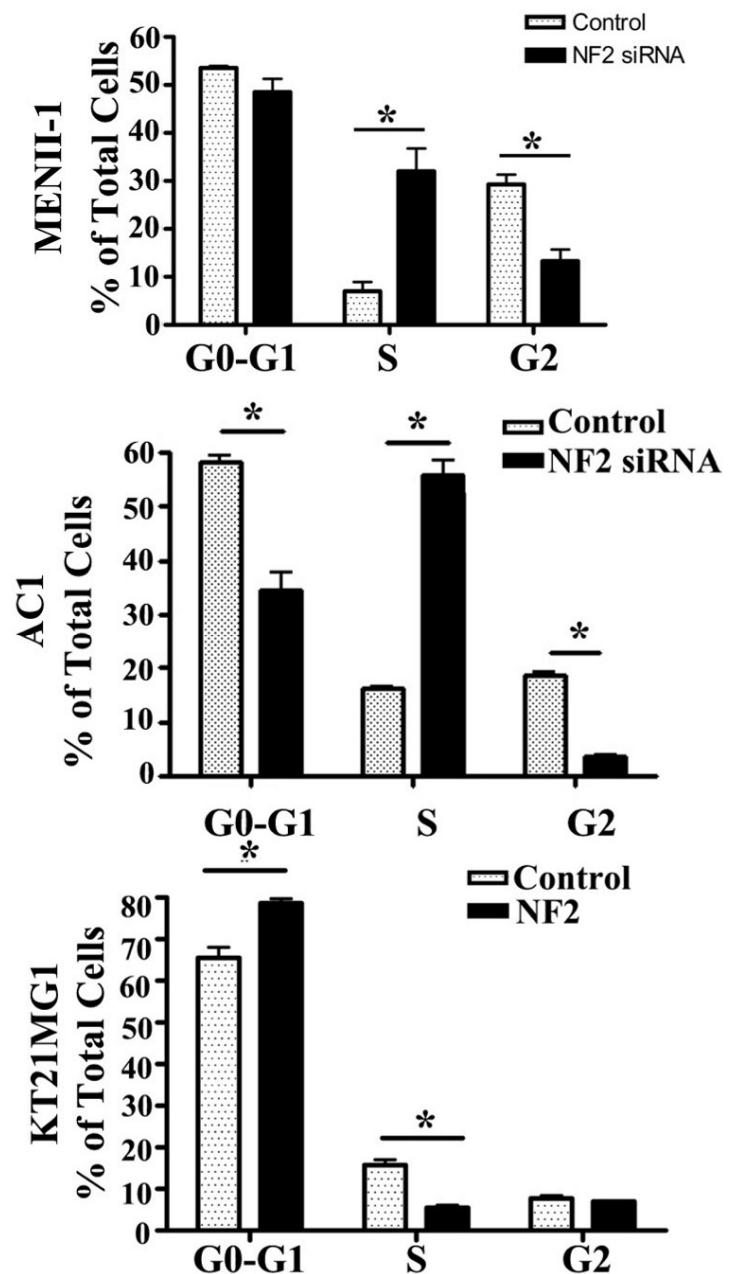


Figure 5. The effect of merlin loss on cell cycle progression. The cell cycle dynamics in the presence and absence of merlin was measured as described in the legend for figure 4. Merlin loss was associated with an increase in the S-phase and decrease in the G0-G1 and G2 phase of the cell cycle.

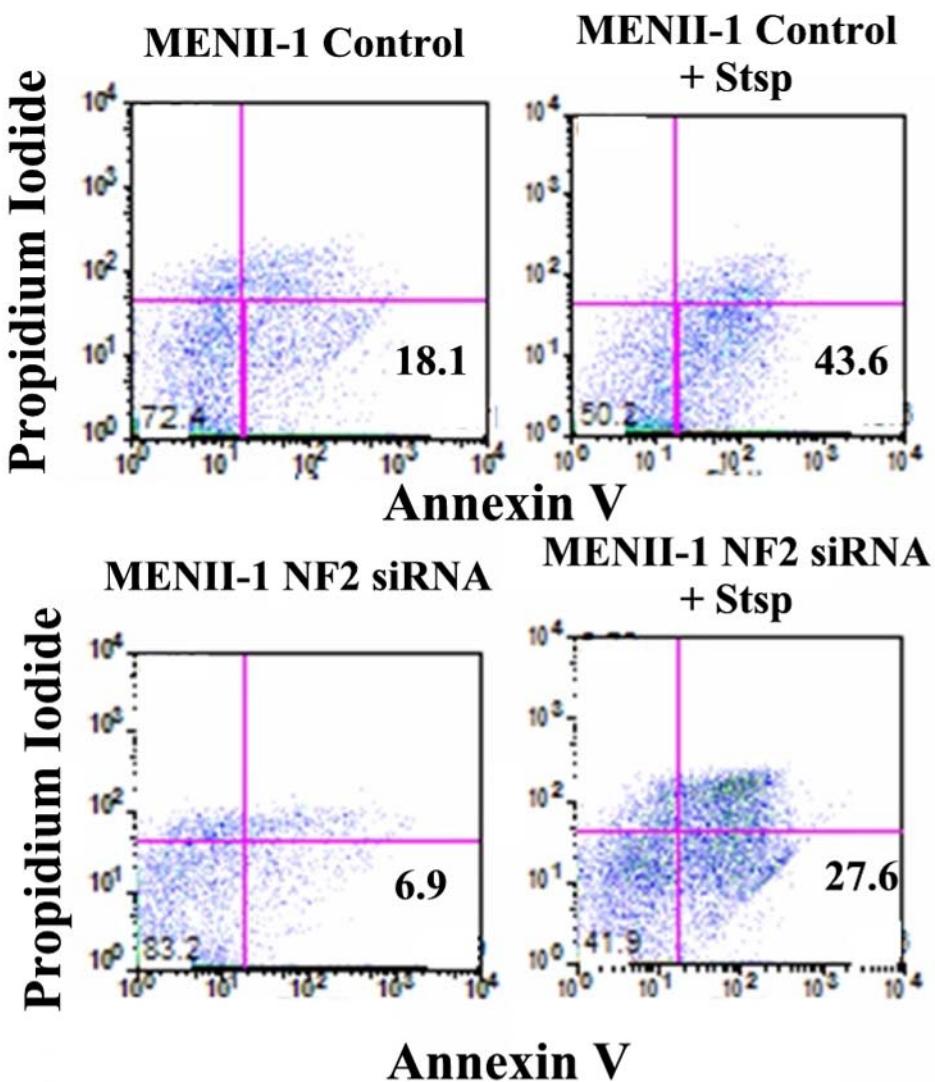


Figure 6. Merlin loss decreases the rate of spontaneous apoptosis and staurosporine (Stsp) mediated apoptosis. MENII-1 control and MENII-1 NF2 siRNA cells were treated with either DMSO or 20 nM staurosporine for 12 h and the apoptotic rates were measured using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen) following manufacturer's directions. The PI negative, Annexin V positive fraction is the apoptotic fraction and the percentage of cells in this fraction are numbered. The apoptotic fraction was decreased in the absence of merlin in the presence and absence of Stsp, suggesting that merlin was affecting apoptosis in addition to cell cycle.

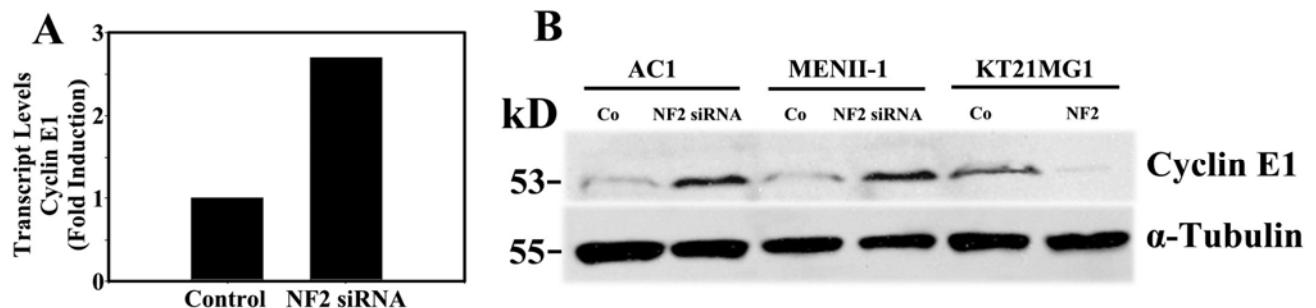


Figure 7. Merlin loss is associated with elevated Cyclin E1 expression. Transcript levels of Cyclin E were evaluated using quantitative PCR in MENII-1-Control and MENII-1-NF2 siRNA cells (A). Fold induction is defined as the ratio of relative transcript levels in NF2 siRNA stably to the relative transcript levels in control cells. A 2.5-fold increase of Cyclin E1 transcript levels in NF2 siRNA cells was observed (A). This was accompanied by upregulation of protein levels of Cyclin E1 as shown by western blot analysis (B). α -Tubulin was used as a loading control.